Exhibit 65

Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions

Shuen-Iu Hung[a,1], Wen-Hung Chung[b,c,1], Shiou-Hwa Jee[d], Wen-Chieh Chen[e], Yun-Ting Chang[f], Woan-Ruoh Lee[g], Shu-Ling Hu[h], Meng-Tse Wu[i], Gwo-Shing Chen[j], Tak-Wah Wong[k], Pa-Fan Hsiao[l], Wei-Hsuan Chen[a], Han-Yu Shih[a], Wu-Hsiang Fang[a], Chun-Yu Wei[a], Yi-Hui Lou[a], Yau-Li Huang[b], Juei-Jueng Lin[m] and Yuan-Tsong Chen[a,n]

The anticonvulsant carbamazepine (CBZ) frequently causes cutaneous adverse drug reactions (cADRs), including maculopapular eruption (MPE), hypersensitivity syndrome (HSS), Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). We reported that SJS/TEN caused by CBZ is strongly associated with the *HLA-B*1502* gene in Han Chinese. Here, we extended our genetic study to different types of CBZ-cADRs (91 patients, including 60 patients with SJS/TEN, 13 patients with hypersensitivity syndrome and 18 with maculopapular exanthema versus 144 tolerant controls). We used MALDI-TOF mass spectrometry to screen the genetic association of 278 single nucleotide polymorphisms (SNPs), which cover the major histocompatibility complex (MHC) region, tumor necrosis factor-alpha, heat shock protein and CBZ-metabolic enzymes, including CYP3A4, 2B6, 2C8, 2C9, 1A2 and epoxide hydrolase 1. In addition, we genotyped 20 microsatellites in the MHC region and performed HLA-typing to construct the recombinant map. We narrowed the susceptibility locus for CBZ-SJS/TEN to within 86 kb flanking the *HLA-B* gene on the extended B*1502 haplotype, and confirmed the association of B*1502 with SJS/TEN [$P_c = 1.6 \times 10^{-41}$, odds ratio (OR) = 1357; 95% confidence interval (CI) = 193.4–8838.3]. By contrast to CBZ-SJS/TEN, *HLA-B*1502* association was not observed in the MPE or HSS groups: MPE was associated with SNPs in the HLA-E region and a nearby allele, *HLA-A*3101* ($P_c = 2.2 \times 10^{-3}$, OR = 17.5; 95% CI = 4.6–66.5), and HSS with SNPs in the motilin gene ($P_c = 0.0064$, OR = 7.11; 95% CI = 3.1–16.5) located terminal to the MHC class II genes. No SNPs in genes involved in CBZ metabolism were associated with CBZ-induced cADRs. Our data suggest that *HLA-B*1502* could contribute to the pathogenesis of CBZ-SJS/TEN, and that genetic susceptibility to CBZ-induced cADRs is phenotype-specific. *Pharmacogenetics and Genomics* 16:297–306

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[a]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, [b]Department of Dermatology, Chang Gung Memorial Hospital, Taipei, Taiwan, [c]Molecular Medicine Program of Taiwan International Graduate Program, Academia Sinica, and the School of Life Sciences, National Yang-Ming University, Taipei, Taiwan, [d]Department of Dermatology, National Taiwan University Hospital, Taipei, Taiwan, [e]Department of Dermatology, Chang Gung Memorial Hospital, Kaohsiung, Taiwan, [f]Department of Dermatology, Veterans General Hospital-Taipei, Taiwan, [g]Department of Dermatology, Taipei Medical University Hospital, Taipei, Taiwan, [h]Department of Dermatology, Cathay General Hospital, Taipei, Taiwan, [i]Department of Dermatology, Chine Medical University Hospital, Taichung, Taiwan, [j]Department of Dermatology, Kaohsiung Medical Univerity Hospital, Kaohsiung, Taiwan, [k]Department of Dermatology, Biochemistry and Molecular Biology & Institute of Clinical Medicine, College of Medicine, National Cheng Kung Univerelty, Tainan, Taiwan, [l]Department of Dermatology, Mackay Memorial Hospital, Taipei, Taiwan, [m]Department of Neurology, Chushang Show-Chwen Hospital, Nantou, Taiwan and [n]Department of Pediatrics, Duke University Medical Centre, Durham, North Carolina 27710, USA

Correspondence and requests for reprints to Professor Yuan-Tsong Chen, Institute of Biomedical Sciences, Academia Sinica, 128, Academia Road, Section 2, Nankang, Taipei, Taiwan
Tel: +11 886 2 2789 9104; fax: +11 886 2 2782 5573;
e-mail: chen0010@ibms.sinica.edu.tw

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Introduction

Although carbamazepine (CBZ) is a commonly prescribed first-line anticonvulsant, it is also a relatively common cause of cutaneous adverse drug reactions (cADRs) [1]. The frequency of CBZ-induced cADRs is between 1 in 1000 and 1 in 10 000 new exposures to the drug in Caucasians [2,3]. The cADRs range from mild maculopapular eruption (MPE), with increasing severity, to hypersensitivity syndrome (HSS), Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). The mortality rate of the latter can be as high as 40% [4]. MPE is characterized by cutaneous fine pink macules and

[1]Both authors contributed equally to this work.

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papules, lesions which usually fade within 1–2 weeks following cessation of drug treatment. HSS is characterized by multi-organ involvement (e.g. hepatitis and nephritis) accompanied by systemic manifestations (e.g. fever, arthragias, eosinophilia and lymphadenopathy) in addition to skin rashes [4]. Skin manifestations of HSS may vary from MPE to exfoliative dermatitis [5]. HSS is also called drug rash with eosinophilia and systemic symptoms. SJS and TEN are characterized by a rapidly developing blistering exanthema of purpuric macules and target-like lesions accompanied by mucosal involvement and skin detachment [6]. According to the clinical morphology, SJS/TEN belong to the group of bullous cADRs, whereas MPE and HSS are non-bullous reactions.

The mechanism by which CBZ causes cADRs is not well understood. Potential defects in the enzymes responsible for bioactivation and detoxification of CBZ have been proposed [1]. Carbamazepine is bioactivated by hepatic cytochrome P450 enzymes, mainly CYP3A4, CYP2B6 and CYP2C8, which generate various potentially reactive metabolites, such as CBZ 10,11-epoxide, 3-hydroxy CBZ, 2-hydroxy CBZ, and CBZ 2,3-epoxide [7,8]. Most of the reactive epoxides are detoxified to non-toxic dihydrodiols by liver microsomal epoxide hydrolase 1 (EPHX1) or to glutathione conjugates by glutathione transferase [9,10]. Previous attempts to identify a genetic defect altering the structure or function of epoxide hydrolase 1 in individuals susceptible to CBZ-induced cADRs, however, were not successful [11,12].

It has also been proposed that immune reactions are involved in the cADRs triggered by CBZ because infiltrating inflammatory cells can be detected in the skin lesions, and rechallenging with the same drug typically shortens the incubation period, resulting in more severe manifestations [13,14]. CD4 + T cells are the major cell type found in the skin lesions of MPE and HSS [15], whereas CD8 + T-cell-mediated cytotoxic responses appear to be the major event in SJS/TEN [16]. There is also evidence supporting the view that cADRs involve major histocompatibility complex (MHC)-dependent presentation of its metabolites for T cell activation [16,17]. Naisbitt *et al.* [15] reported that CBZ might be presented by MHC class II expressed on the surface of antigen-presenting cells to the T-cell receptor of CD4 + T cells of patients with CBZ-induced HSS [15].

We recently reported that, in Han Chinese, the *HLA-B*1502* gene is strongly associated with CBZ-induced SJS and TEN [18]. In the present study, we sought to extend our genetic association study to other types of cADRs induced by CBZ using a comprehensive genetic screen of markers in the MHC region, as well as genes encoding CBZ metabolic enzymes. In addition, we performed fine mapping to further investigate the susceptibility locus for CBZ-induced SJS/TEN.

Materials and methods

Study population

From 1997 to 2004, 91 individuals who fulfilled diagnostic criteria for CBZ-induced MPE, HSS, SJS or TEN agreed to participate in the study. Eighty-eight of the 91 patients were recruited from Chang Gung Memorial Hospital Health System, Veterans General Hospital, National Taiwan University Hospital, Cathay General Hospital and Chushang Show-Chwan Hospital, Taiwan. We recruited patients primarily from inpatients who had more severe diseases. The remaining three patients were referred by physicians from Hong Kong (two patients) and the USA (one patient). The latter three all had CBZ-SJS. All patients (including the three from Hong Kong and the USA) and controls (see below) were Han Chinese or Chinese descendants.

The 91 patients included 44 patients with CBZ-SJS/TEN who we previously reported [18], and 16 additional patients with SJS/TEN, as well as 13 patients with hypersensitivity syndrome and 18 with maculopapular exanthema. All patients were assessed by two dermatologists who reviewed photographs, pathological slides and medical records. Diagnostic criteria for SJS/TEN were based on the clinical morphology defined by Roujeau [6]. We defined 'SJS' as a skin detachment of less than 10% of total body-surface area, 'overlap of SJS and TEN' as skin detachment of 10–30%, and 'TEN' as skin detachment greater than 30%. The criteria for HSS in this study were skin rash, plus two of the following symptoms: fever, lymphadenopathy and haematologic abnormalities (e.g. eosinophilia, atypical lymphocytosis) with involvement of at least one internal organ (e.g. hepatitis, pneumonitis, myocarditis, pericarditis, nephritis) [2,5,19]. SJS and TEN are bullous cADRs and are considered to be variants of the same disease. Therefore, they were analysed together as a group. MPE and HSS are non-bullous and it is less clear whether they are from a single disease spectrum. They were therefore analysed as a group and also analysed separately as two entities. In all enrolled cases, CBZ was regarded as the offending drug if the onset of cADRs symptoms occurred within the first 2 months of exposure and the symptoms resolved upon withdrawal of the drug. Patients with an absence of symptoms after re-exposure to CBZ were excluded.

The control group was the 144 consecutive patients who received CBZ for at least 3 months without evidence of adverse reactions. These tolerant patients were recruited from the Neurology Clinic of the same regional hospitals where cADRs patients were recruited. In addition, 93 healthy subjects were randomly selected from a biobank under a nationwide population study, in which 3312 Han

Chinese descendants were recruited based on the geographical distribution across Taiwan. There was no self-report of adverse events in any of these 93 subjects. The study was approved by the institutional review board, and informed consent was obtained from all of the participants.

DNA isolation and genotyping

Genomic DNA was isolated using the PUREGENE DNA purification system (Gentra Systems, Minneapolis, Minnesota, USA).

Short tandem repeat polymorphism (STRP) genotyping

Twenty highly polymorphic microsatellite markers located in the MHC region were selected from the NCBI database (i.e. D6S258, D6S2972, D6S510, D6S265, D6S388, D6S2814, HLAC-CA, HLABC-CA, MIB, MICA, TNFd, BAT2-CA, D6S273, D6S1615, DQCAR, G51152, D6S2414, D6S1867, D6S1560 and D6S1583). The average heterozygosity of markers was 0.702 with an estimated 230 kb of spacing. Primers were designed based on oligonucleotide sequences reported within the database. Polymerase chain reaction (PCR) for genotyping was performed in a 5-μl volume containing 10 ng of genomic DNA and 0.33 μM of each primer by using GeneAmp 9700 thermocyclers (Applied Biosystems, Foster City, California, USA). Up to six products of appropriate size and carrying a fluorescent label were pooled for capillary gel electrophoresis. The size of polymorphic amplicons was determined by ABI 3730 DNA sequencer (Applied Biosystems) using the LIZ500 size standard as an internal size standard. Allele sizing was calculated using the GENMAPPER program version 3.0 (Applied Biosystems). Allele calling and binning were performed using the SAS program (SAS Institute, Cary, North Carolina, USA). Three CEPH control individuals (1331-01, 1331-02, 1347-2) and H_2O were included in all genotyping experiments for quality control purposes.

Single nucleotide polymorphisms (SNP) genotyping on MHC region and metabolic enzymes for CBZ

A total of 379 SNPs were selected from the SNP database (dbSNP: build 123) of the National Center for Biotechnology Information for genotyping [20]. These included 220 SNPs from 4 Mb of the MHC region on chromosome 6p21.3 and 159 SNPs selected from genes encoding drug metabolizing enzymes. The 220 SNPs selected in the MHC region included 201 SNPs reported by Walsh *et al.* [21], and additionally included SNPs of tumor necrosis factor (TNF)-alpha and the heat shock proteins. We especially included rs1800629, TNF-308A allele, which is reported to be associated with CBZ-HSS [22], and rs2227956 of HspA1L (heat shock 70 kDa protein1-like), which is reported to be associated with abacavir hypersensitivity [23]. The average space of selected SNPs in the MHC region was approximately 20 kb. Another 159 SNP set contained genes potentially involved in bioactivation or detoxification of CBZ metabolism, including CYP3A4, CYP2B6, CYP2C8, CYP2C9, CYP1A2 and EPHX1.

SNP genotyping was performed using high-throughput MALDI-TOF mass spectrometry. Briefly, primers and probes were designed using SpectroDESIGNER software (Sequenom, San Diego, California, USA). Multiplex PCRs were performed, and unincorporated dNTPs were dephosphorylated using shrimp alkaline phosphatase (Hoffman-LaRoche, Basel, Switzerland) followed by primer extension. The purified primer extension reaction was spotted onto a 384-element silicon chip (SpectroCHIP, Sequenom), analysed in the Bruker Biflex III MALDI-TOF SpectroREADER mass spectrometer (Sequenom) and the resulting spectra processed with SpectroTYPER (Sequenom).

Of the 379 SNPs selected from the database, 101 SNPs were excluded from further analysis due to the non-polymorphism, low successful rate (less than 0.7), and/or departure from Hardy–Weinberg equilibrium ($P < 0.001$) in our control group. The remaining 278 SNPs, including 88 SNPs in metabolic pathway for CBZ and 190 SNPs in the MHC region, were used for the statistical analyses and the *P*-values of association were adjusted by using Bonferroni correction.

HLA genotyping

HLA alleles *A*, *B*, *C* and *DRB1* were determined by sequence-specific oligonucleotide reverse lineblots (DYNAL Biotech Ltd, Bromborough, UK) [24]. Potential ambiguities were resolved by sequencing-based typing [25]. The primers used for PCR amplification of DNA fragments of *HLA-A*, *B* and *C* genes were: (i) Bin1-TA-M13F, Bin1-CG-M13F and Bin3-M13R for *HLA-B* alleles; (ii) Ain1-A-M13F, Ain1-G-M13F, Ain1-T-M13F and Ain3–62-M13R for *HLA-A* alleles; and (iii) 5Cln1-61 and 3BCln3-12 for *HLA-C* alleles [26]. The *DRB1* alleles were separated into eight groups: DR1, DR2, DR3/11/6, DR4, DR7, DR8/12, DR9, DR10 for sequencing-based typing as described [27]. Genotyping for MHC class I chain-related gene A (MICA) was carried out by DNA sequencing on its exons 2, 3, 4 and 5 [25]. The primers used for PCR amplification of the DNA fragment of the *MICA* gene were: forward primer: 5′-CGTTGTTGTCCCTTTGCCCGTGTGC-3′ and reverse primer: 5′-GATGCTGCCCCATTCCCTTCCCAA-3′. The sequence data were analysed by SeqScape v2 (Applied Biosystems).

Statistical analysis

Comparisons of allele or genotype frequencies between groups were performed using Fisher's exact tests. All *P*-values were two-tailed. $P < 0.05$ was considered to be statistically significant. An allelic association screen was

carried out by the Cochran–Armitage Trend test for each SNP or STRP marker [28]. To test the association of haplotype frequencies, the composite haplotype method and haplotype trend regression of Helix Tree software version 3.0.0 were used. The linkage disequilibrium between two loci was analysed by using Expectation/Maximization (EM) method provided by Helix Tree software version 3.0.0 (Golden Helix Inc., Bozeman, Minnesota, USA). To achieve sufficient power to identify loci associated with different clinical manifestations, the corrected P (P_c) values were adjusted by using Bonferroni's correction for multiple comparisons (278 for SNP assays, 17 for HLA-A, 40 for HLA-B, 19 for HLA-C, 30 for HLA-DRB1, 25 for MICA, and 20 for HLABC-CA). Odds ratios (ORs) were calculated using Haldane's modification, which adds 0.5 to all cells to accommodate possible zero counts [29].

Results

Characteristics of patients and controls

Based on the clinicopathologic features of cADRs, our patients could be divided into bullous and non-bullous groups: (i) bullous: CBZ-SJS/TEN ($n = 60$; 54 with SJS, five with overlapping SJS/TEN, one with TEN) and (ii) non-bullous: CBZ-MPE/HSS ($n = 31$; 18 with MPE, 13 with HSS). The non-bullous group was further separated into two subgroups (MPE and HSS) for data analysis. Clinical manifestations and demographic variables of the 91 patients and 144 tolerant controls are summarized in Table 1. The onset of symptoms for all patients with cADRs occurred within the first 2 months of CBZ exposure. The mean duration of CBZ exposure was longer in HSS patients than in SJS/TEN. Three patients with SJS/TEN had a second attack within 2 days of re-exposure; one of them developed TEN and died during the second attack. All 60 patients with SJS/TEN had widespread purpuric rash with blisters and mucosal involvement. In addition to maculopapular exanthema, all 13 patients with HSS had symptoms of multi-organ or systemic involvement.

One hundred and forty-four patients who had been on CBZ for at least 3 months (mean 75.03 months) with no self-report of adverse events were used as tolerant controls. Their demographic variables are shown in Table 1. Note that the CBZ-tolerant controls received higher doses of CBZ (mean dosage 793.8 mg/day) and yet no adverse drug reactions were observed.

Fine mapping of the major histocompatibility complex region for genetic susceptibility to CBZ-SJS/TEN

To confirm our previous observation of the genetic association between *HLA-B*1502* and CBZ-SJS/TEN, and to further define the susceptibility region, we genotyped 20 STRP markers and 190 SNPs in the MHC region. Nine STRP markers located between D6S2814 and D6S2414 (physical map 30.8–32.9 Mb, chromosome 6) showed significant association (P-values $< 10^{-6}$; Fig. 1). In particular, HLABC-CA near *HLA-B* showed the strongest association ($P = 3.4 \times 10^{-19}$; Fig. 1), followed by D6S1615, a marker closed to *HLA-DRB1* locus.

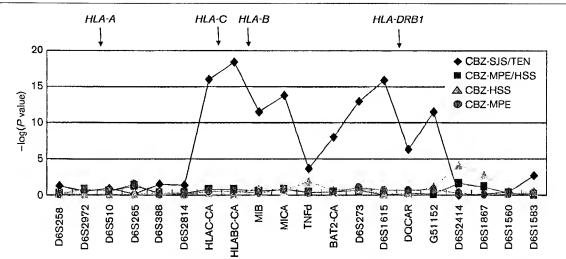
Thirty-seven SNPs showed P-values of less than 0.001 (Fig. 2a). Among them, seven SNPs located between HLA-DRA and HLA-C (physical position 31.3–32.5 Mb, chromosome 6) showed strong associations ($P < 10^{-10}$) (Fig. 2a). The three most important SNPs were rs3130690, rs2848716 and rs750332, located near *HLA-B*, *MICA* and *BAT3* genes (Table 2). The rs3130690, an intergenic SNP with 36 kb telomeric to the *HLA-B* locus, demonstrated the strongest association with CBZ-induced SJS/TEN ($P_c = 1.29 \times 10^{-39}$) (Table 2). The T allele of the rs3130690 SNP was present in 95% (57/60) of CBZ-SJS/TEN patients, but only in 6.9% (10/144) of

Table 1 Demographic variables, dosage and duration of carbamazepine (CBZ) exposure, and clinical characteristics in CBZ-induced cutaneous adverse reactions and CBZ-tolerant controls

	Bullous cADRs	Non-bullous cADRs ($n = 31$)		
	SJS/TEN ($n = 60$)	HSS ($n = 13$)	MPE ($n = 18$)	Tolerant controls ($n = 144$)
Sex, *n* (%)				
Male	33 (55)	9 (69.2)	8 (44.4)	75 (52.1)
Female	27 (45)	4 (30.8)	10 (55.6)	69 (47.9)
Age (years), mean (range)	43.4 (5–80)	51.5 (6–83)	45.9 (7–84)	35.7 (5–79)
CBZ exposure, mean (range)				
Dosage (mg/day)	332.6 (100–600)	420 (300–600)	423.1 (200–800)	793.8 (100–1500)
Duration	15.1 (2–49) days	32.7 (15–54) days	22.4 (7–55) days	75.03 (3–2875) months
Cutaneous features, *n* (%)				
Blister or epidermal detachment	60 (100)	0 (0)	0 (0)	Not observed
Mucosal erosions	60 (100)	3 (23)	0 (0)	Not observed
General and laboratory, *n* (%)				
High fever (>38.5°C)	41 (68.3)	12 (92.3)	11 (61.1)	Not observed
Eosinophil count >1000/mm^3	4 (6.7)	10 (76.9)	4 (22.2)	Not observed
Atypical lymphocytosis	8 (13.3)	7 (53.8)	3 (16.7)	Not observed
Abnormal liver function	7 (11.7)	11 (84.6)	0 (0)	Not observed
Abnormal renal function	1 (1.7)	2 (15.4)	0 (0)	Not observed

SJS/TEN, Stevens–Johnson syndrome/toxic epidermal necrolysis; HSS, hypersensitivity syndrome; MPE, maculopapular exanthema.

Fig. 1



Association screen of 20 short tandem repeat polymorphism (STRP) markers in the major histocompatibility complex (MHC) region with carbamazepine (CBZ)-induced cutaneous adverse drug reactions. On the *x*-axis, 20 STRP markers in the MHC region are ordered by their physical positions (29.9–33.9 Mb) on chromosome 6p21.3. On the *y*-axis, the $-\log_{10} P$ values were calculated by comparison of the allele frequencies between the patients and tolerant group using the Cochran–Armitage exact trend test. Genotyping data of four groups of patients are presented: diamond symbols: CBZ-induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN); square symbols: CBZ-induced maculopapular eruption (MPE)/hypersensitivity syndrome (HSS); triangle symbols: CBZ-induced HSS; and circle symbols: CBZ-induced MPE.

tolerant controls [OR = 254.6; 95% confidence interval (CI) = 70.4–901.9] for TT/TG genotype versus GG genotype) (Fig. 3). To verify the results of our association screen, we also genotyped 93 healthy subjects randomly selected from the general population [30]. A strong association between SNPs and CBZ-induced SJS/TEN was replicated in the MHC region, especially rs3130690, nearby the *HLA-B* locus (data not shown).

We further genotyped the individual *HLA-A, B, C, DRB1* and *MICA* alleles. The *HLA-B*1502* allele was present in 98.3% (59/60) of CBZ-SJS/TEN patients, whereas only 4.2% (6/144) of the tolerant controls were positive for the allele ($P_c = 1.6 \times 10^{-41}$, OR = 1357; 95% CI = 193.4–8838.3) (Table 3 and Fig. 3). The only CBZ-SJS patient who did not have the *B*1502* allele, had instead, another HLA-B15 allele: *HLA-B*1558*. In addition to the positive association, we also found that *HLA-B*4001* was negatively associated with CBZ-SJS/TEN ($P_c = 2.6 \times 10^{-4}$, OR = 0.16; 95% CI = 0.1–0.4) (Table 3). The *HLA-Cw*0801* and *MICA*019* alleles flanking the *HLA-B*1502* showed strong linkage disequilibrium with *HLA-B*1502*, and were present in 93.3% (56/60) and 95% (57/60) of SJS/TEN patients, respectively. An extended B*1502 haplotype formed by polymorphic alleles (A*1101-Cw*0801-HLABC-CA*119-rs3130690T-B*1502-MICA*019-DRB1*1202) had a strong association with CBZ-induced SJS/TEN. The recombinant map of Cw*0801-HLABC-CA*119-rs3130690T-B*1502-MICA*019 defined the susceptible region within 86 kb (i.e. between the T allele of rs3130690 and *MICA*019*) flanking the *B*1502* gene in the 4 Mb MHC region (Fig. 3). Within this 86-kb region, *HLA-B* is the only known gene. Taken together, the data suggested that one or more alleles in the vicinity of the *HLA-B* locus, particularly *B*1502* itself, participate in the pathogenesis of CBZ-induced SJS/TEN.

Association screen for candidate gene SNPs with CBZ-MPE/HSS

When MPE and HSS were grouped together as non-bullous cADRs, no STRP markers showed association, and only six SNPs had *P*-values less than 0.01 (Figs 1 and 2b). The three most significant SNPs are listed in Table 2, including rs1264511 located near HLA-E, rs1042389 located on the 3′-untranslation region of CYP2B6, and rs2894342 located in the promoter region of the motilin (*MLN*) gene (Table 2). However, the *P*-values became non-significant after correcting for multiple testing (278 SNP assays) was performed.

In HLA-typing, *HLA-A*3101* showed an association with MPE/HSS ($P_c = 0.0021$) (Table 3). *HLA-A*3101* was present in 25.8% (8/31) of patients with MPE/HSS, but only in 2.8% (4/144) of tolerant controls (OR = 12.17; 95% CI = 3.6–41.2).

Association between *MLN* polymorphisms and HSS

When genetic analysis was performed separately for HSS and MPE, one STRP marker, D6S2414 (physical map: 32.9 Mb, chromosome 6) showed an association with

Table 2 **Association between the three most significant single nucleotide polymorphism (SNP) alleles and carbamazepine (CBZ)-induced cutaneous adverse reactions**

Groups	Reference SNP	Chromosomal position	Gene symbol	Location	Allele type		Allele frequency		*P*-value	P_c value	Odds ratio (95% confidence interval)
							Case	Controls [a]			
SJS/TEN (n=60)	rs3130690	6:31393914	Near HLA-B	Intergene	T/G	T	0.683	0.045	4.66×10^{-42}	1.29×10^{-39}	45.65 (23.34–89.13)
	rs2848716	6:31495946	MICA	3′-UTR	C/G	C	0.691	0.246	7.58×10^{-17}	2.10×10^{-14}	6.86 (4.29–10.96)
	rs750332	6:31715029	BAT3	Intron	C/T	C	0.508	0.139	3.04×10^{-14}	8.45×10^{-12}	6.41 (3.94–10.44)
MPE/HSS (n=31)	rs1264511	6:30521951	Near HLA-E	Intergene	G/C	G	0.274	0.108	0.001685	NS	3.13 (1.61–6.09)
	rs1042389	19:46215993	CYP2B6	3′-UTR	C/T	C	0.516	0.299	0.001696	NS	2.51 (1.44–4.36)
	rs2894342	6:33882372	MLN	Promoter	A/C	A	0.258	0.108	0.00342	NS	2.88 (1.47–3.66)
HSS (n=13)	rs2894342	6:33882372	MLN	Promoter	A/C	A	0.462	0.108	0.0000232	0.0064	7.11 (3.07–16.51)
	rs2075800	6:31885925	HSPA1L	Coding	G/A	G	0.923	0.622	0.001173	NS	7.31 (1.87–28.37)
	rs2395402	6:33861651	LEMD2	Intron	G/A	G	0.423	0.160	0.002381	NS	3.86 (1.89–8.80)
MPE (n=18)	rs1264511	6:30521951	Near HLA-E	Intergene	G/C	G	0.333	0.108	0.0007862	NS	4.15 (1.91–9.02)
	rs1059510	6:30565711	HLA-E	Coding	T/C	T	0.472	0.243	0.005176	NS	2.79 (1.39–5.60)
	rs986475	6:31664688	LST1	5′-UTR	C/T	C	0.278	0.108	0.007597	NS	3.19 (1.43–7.15)

[a]144 tolerant-controls were genotyped. The association of allele frequencies was examined by Fisher's exact test and the P_c values were adjusted by using Bonferroni's correction for multiple comparisons of 278 SNP assays. NS, not significant (P>0.05). SJS/TEN, Stevens–Johnson syndrome/toxic epidermal necrolysis; MPE, maculopapular exanthema; HSS, hypersensitivity syndrome.

CBZ-induced HSS ($P = 6.25 \times 10^{-5}$, Fig. 1). In addition, several SNPs located in the MHC class II terminal region, around the motilin gene (physical map: 33.8 Mb), showed associations with HSS (Table 2 and Fig. 2c). The most significant SNP rs2894342 was located in the promoter of the *MLN* gene, and its A allele had an increased risk for CBZ-HSS ($P_c = 0.0064$, OR = 7.11; 95% CI = 3.1–16.5) (Table 2). The next most significant SNP was a nonsynonymous SNP (rs2075800 of HSPA1L, heat shock 70 kDa protein1-like) involving an A to G transition which leads to an amino acid change of lysine to glutamate at residue 602. The third most significant SNP was rs2395402, which was located in the intron of the LEM domain containing 2 (LEMD2) gene, 21 kb telomeric to rs2894342 of the *MLN* gene (Table 2). However, the *P*-values for the latter two SNPs became non-significant after Bonferroni's correction.

None of the *HLA-A*, *B*, *C* and *DRB1* alleles had significant associations with CBZ-induced HSS (Table 3).

Association between *HLA-A*3101* and MPE

STRP or SNP markers in the *HLA-B* or *MLN* region on chromosome 6p21.3 showed no association with MPE (Figs 1 and 2d). Instead, two SNPs near the HLA-E region (physical position 30.5 Mb) and one SNP in the 5′-untranslation region of the leukocyte specific transcript 1 (LST1) gene showed associations with MPE (Fig. 2d and Table 2, $P = 0.00078–0.0075$). The two SNPs in the HLA-E region were: rs1264511, an intergenic SNP located near HLA-E and rs1059510 located in HLA-E gene (Table 2). HLA-genotyping further revealed that the *HLA-A*3101* allele, approximately 530 kb telomeric to HLA-E, was present in 33.3% (6/18) of MPE patients but only in 2.8% (4/144) in the tolerant control group ($P_c = 2.2 \times 10^{-3}$, OR = 17.5; 95% CI = 4.6–66.5) (Table 3).

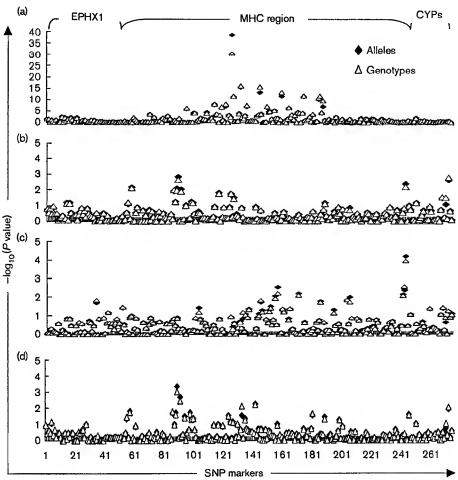
Association screen for SNPs in drug metabolizing enzymes

Genotyping of 88 informative SNPs in genes that are potentially involved in metabolic pathways for CBZ, including CYP3A4, CYP1A2, CYP2C8, CYP2B6, CYP2C9 and EPHX1, in various types of CBZ-induced cADRs, showed that only one SNP, rs1042389, in the 3′-untranslation region of CYP2B6, had a weak association with CBZ-MPE/HSS ($P = 0.0016$, Table 2 and Fig. 2b). The *P*-value became non-significant after Bonferroni's correction, suggesting that the genetic polymorphisms of genes encoding drug metabolizing enzymes have no significant association with CBZ-induced cADRs, regardless of the type of cADRs.

Discussion

This study confirmed our previous observation that *HLA-B*1502* is strongly associated with CBZ-SJS/TEN in Han

Fig. 2



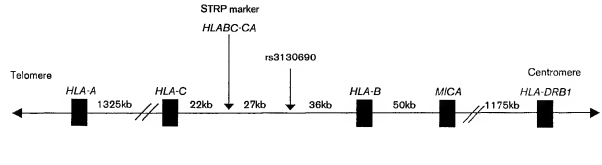
Screening of 278 candidate single nucleotide polymorphisms (SNPs) for association with (a) carbamazepine (CBZ)-induced Stevens–Johnson syndrome/toxic epidermal necrolysis, (b) CBZ-induced maculopapular eruption (MPE)/hypersensitivity syndrome (HSS), (c) CBZ-induced HSS and (d) CBZ-induced MPE. On the *x*-axis, 278 SNPs are ordered by their chromosomal positions, including 58 SNPs of epoxide hydrolase 1 (EPHX1) on chromosome 1, 190 SNPs in the major histocompatibility complex (MHC) region on chromosome 6, and 30 SNPs of CYP3A4, 2C8, 2C9, 1A2 and 2B6. On the *y*-axis, the $-\log_{10}$ *P*-values were calculated by comparison of the allele (diamond symbols) or genotype (triangle symbols) frequencies between the patients and tolerant group using the Cochran–Armitage exact trend test.

Chinese [18]. We increased the number of study patients from the original 44 to 60 and included patients from a wide geographical distribution (Taiwan, Hong Kong, China and the USA). However, all patients enrolled were Chinese or Chinese descendants. The fact that the *HLA-B*1502* allele was present in low frequency in Caucasians may explain the low incidence of CBZ-SJS/TEN in Caucasians [31,32].

It is interesting to note that the only CBZ-SJS/TEN patient who did not have the *HLA-B*1502* gene had *HLA-B*1558*, another HLA-B15 variant. This variant allele was present in low frequency in our population (approximately 0.9%) and was not detected in any of the 144 tolerant patients. The detection of *B*1558* in one of our patients may imply that *B*1558* shares a similar structural feature with *B*1502* for triggering the immune reaction of SJS caused by CBZ.

Fine recombinant genetic mapping on the extended *HLA-B*1502* haplotype (A*1101-Cw*0801-HLABC-CA*119-rs 3130690T-B*1502-MICA*019-DRB1*1202) further narrowed

Fig. 3



SJS/TEN	70%	93.3%	90%	95%	98.3%	95%	68.3%
Tolerant	46.5%	13.9%	4.2%	6.9%	4.2%	13.2%	16%
P value	0.003	4×10^{-28}	2×10^{-35}	4×10^{-36}	4×10^{-43}	5×10^{-30}	7×10^{-13}
P_c value	N.S	8×10^{-27}	4×10^{-34}	1×10^{-35}	2×10^{-41}	1×10^{-28}	2×10^{-11}
Odds ratio	2.7	86.8	207.0	254.6	1357	125	11.4
(95% C.I.)	(1.4–5.1)	(29.3–254.6)	(65.0–659.1)	(70.4–901.9)	(193.4–8838.3)	(37.3–412.4)	(5.64–22.9)

Susceptible region

Susceptible region for carbamazepine (CBZ)-induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) on the extended *HLA-B*1502* haplotype. Schematic map of 4 Mb major histocompatibility complex region on chromosome 6p21.3 shows the relative positions of *HLA-A, B, C, DRB1* and *MICA* genes, STRP marker (HLABC-CA), and SNP marker (rs3130690). The percentage of presence of the specific allele in CBZ-SJS/TEN patients and tolerant-controls is shown. The susceptible region should be within 86 kb (i.e. between T allele of rs3130690 and *MICA*019*). Within this region, *HLA-B*1502* is the only known gene and shows the strongest association with CBZ-induced SJS/TEN. NS, Not significant ($P>0.05$).

Table 3 Association of HLA alleles with carbamazepine (CBZ)-induced cutaneous adverse reactions

	Tolerant controls ($n=144$)	SJS/TEN ($n=60$)	MPE/HSS ($n=31$)	HSS ($n=13$)	MPE ($n=16$)
HLA-A					
**1101*	67	42 [NS; 2.68 (1.4–5.1)]	12 [NS; 0.73 (0.3–1.6)]	6 [NS; 0.99 (0.3–3)]	6 [NS; 0.56 (0.2–1.6)]
**2402*	41	5 [0.026; 0.23 (0.1–0.6)]	9 [NS; 1.03 (0.5–2.4)]	3 [NS; 0.75 (0.2–2.7)]	6 [NS; 1.26 (0.5–3.5)]
**3101*	4	1 [NS; 0.59 (0.1–4.1)]	6 [0.0021; 12.17 (3.6–41.2)]	2 [NS; 6.36 (1.2–33.9)]	6 [2.2×10^{-3}; 17.5 (4.6–66.5)]
HLA-B					
**1502*	6	59 [1.6×10^{-41}; 1357 (193.4–8838.3)]	1 [NS; 0.77 (0.1–5.1)]	0 [NS; 0.79 (0.1–6.6)]	1 [NS; 1.35 (0.2–9.3)]
**4001*	59	6 [2.6×10^{-4}; 0.16 (0.1–0.4)]	10 [NS; 0.69 (0.3–1.5)]	3 [NS; 0.43 (0.1–1.5)]	7 [NS; 0.92 (0.4–2.4)]
HLA-Cw					
**0102*	54	8 [6.6×10^{-3}; 0.26 (0.1–0.6)]	14 [NS; 1.4 (0.6–3.0)]	5 [NS; 1.04 (0.3–3.2)]	9 [NS; 1.67 (0.6–4.3)]
**0801*	20	56 [7.8×10^{-27}; 86.8 (29.3–254.6)]	2 [NS; 0.43 (0.1–1.8)]	0 [NS; 0.23 (0–2.3)]	2 [NS; 0.78 (0.2–3.3)]
HLA-DRB1					
**0405*	25	1 [0.03; 0.08 (0.01–0.5)]	8 [NS; 1.66 (0.7–4.1)]	1 [NS; 0.40 (0–2.5)]	7 [NS; 3.03 (1.1–8.4)]
**1202*	23	41 [2.3×10^{-11}; 11.4 (5.6–22.9)]	5 [NS; 1.01 (0.4–2.8)]	3 [NS; 1.56 (0.4–5.8)]	2 [NS; 0.66 (0.2–2.6)]

Data are genotype, *n* of positive subjects [P_c value; odds ratio (95% confidence interval)]. The association of HLA-alleles was examined by Fisher's exact test and the P_c values were adjusted by using Bonferroni's correction for multiple comparisons (17 for HLA-A, 40 for HLA-B, 19 for HLA-C and 30 for HLA-DRB1). NS, Not significant ($P>0.05$). SJS/TEN, Stevens–Johnson syndrome/toxic epidermal necrolysis; MPE, maculopapular exanthema; HSS, hypersensitivity syndrome.

down the susceptibility region for CBZ-SJS/TEN to within 86 kb flanking the *HLA-B* gene. In this region, *HLA-B* is the only gene present. Thus, it is most likely that *B*1502* itself is the susceptibility gene for CBZ-SJS. Furthermore, it has been proposed that the molecular mechanism causing SJS/TEN involves CD8+ cytotoxic T-cell-mediated cell death [16]. The *HLA-B* gene can elicit immune responses by presenting endogenous antigens to the cytotoxic T cells and it has been proposed that the drug and/or its metabolites may bind to the peptides which are then presented by the *HLA-B* allele and recognized by the specific T cells [33,34]. Our

cytotoxic assays also supported the view that *HLA-B*1502* is functionally involved in the cytotoxic response mediated by the activated T cells in CBZ-induced SJS (unpublished data). The recent findings of *HLA-B*5701* in abacavir hypersensitivity [35] and *HLA-B*5801* in allopurinol-induced severe cADRs [26] further support the important role of the *HLA-B* gene in these serious drug-induced adverse conditions.

SJS and TEN are cADRs characterized by bullous lesions. We observed a single patient who developed a bullous fixed drug eruption while on CBZ. Interestingly, this patient was also positive for *HLA-B*1502* (data not shown). However, the present study clearly showed that non-bullous cADRs caused by CBZ are not linked to the *HLA-B*1502* marker, suggesting that the genetic association of CBZ-cADRs is phenotype-specific. It should be pointed out that our previous study of allopurinol induced cADRs showed that both SJS/TEN and HSS were associated with a same *HLA-B* allele [26]. However, in this study, CBZ-HSS is not linked to the *HLA-B* marker as SJS/TEN. At the present time, we do not know why this discrepancy occurs. Other genetic or environmental factors may contribute and lead to various spectrum of adverse reactions caused by different drugs.

Although there is no direct evidence that MPE and HSS are the same disease spectrum with differences in severity, HSS and MPE may be grouped together as non-bullous cADRs because of the similarity of the cutaneous manifestation [2,5,19]. However, the present study suggests that HSS and MPE are two disease entities because they are linked to different genetic markers: CBZ-MPE was associated with *HLA-A*3101*, and CBZ-HSS with the motilin gene polymorphisms in the MHC class II terminal region (Figs 1 and 2 and Tables 2 and 3). We did not observe the association of CBZ-HSS with the TNF2 allele (rs1800629; TNF-308A allele; physical position: 31.6 Mb; Fig. 2c) or the ancestral haplotype 8.1 on the MHC, as reported previously in a study of CBZ-induced HSS in Caucasians [22]. This could be due to different study populations or different criteria in delineating the clinical phenotypes. In the present study, the SJS/TEN patients outnumber the patients with MPE as a reflection of the recruitment process because we recruited patients primarily from inpatients who had more severe diseases. However, in Han Chinese, patients with CBZ-SJS/TEN were indeed more frequently seen than CBZ-HSS (data not shown). Because of the small numbers of the patients enrolled in this study, further studies with a larger sample size will be needed to confirm this initial observation.

Naisbitt *et al.* [15] cloned T cells of patients with CBZ-MPE/HSS and observed that T cells may recognize CBZ depending on the presence of HLA class II (DR/DQ)-matched antigen-presenting cells. Interestingly, in our SNP and STRP screen and HLA typing data, we observed associations between CBZ-induced HSS and polymorphisms markers located terminal to MHC class II region. However, the association of *HLA-A*3101* with MPE appears in disagreement with the observation that CD4+ T cells are the major cell type found in the skin lesions of MPE [15]. This could suggest that the gene associated with MPE lies in the vicinity of the *HLA-A* locus which is in linkage disequilibrium with *HLA-A*3101*.

In conclusion, our data show that genetic susceptibility to CBZ-induced cADRs is phenotype-specific. The susceptibility gene for CBZ-induced SJS/TEN lies within an 86 kb region flanking the HLA-B locus and *HLA-B*1502* itself could be directly involved in the pathogenesis of CBZ-induced SJS/TEN. The tight association of *HLA-B*1502* and CBZ-SJS/TEN provides a plausible basis for the development of such a test to identify individuals at risk for this potentially life-threatening condition caused by CBZ in Han Chinese, as well as for a further increased understanding of the pathogenesis of the clinical syndrome.

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